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Citrullination preferentially proceeds in glomerular Bowman's capsule and increases in obstructive nephropathy

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Background. Peptidylarginine deiminases (PADs) are a group of posttranslational modification enzymes that citrullinate (deiminate) protein arginine residues, yielding citrulline residues. Citrullination of arginine residues abolishes their positive charge, markedly altering their structure. We undertook this study to investigate the actions of PADs in the kidney.

Methods. In male rats, we ligated the unilateral ureter, then analyzed the obstructed and contralateral kidneys 1 week later. Controls were rats simultaneously given sham operations. In another experiment, we ligated unilateral ureters of eight rats, four of which received a ureter-bladder anastomosis 1 week later. These rats were subjected to histologic examinations 5 weeks after unilateral ureteral obstruction (UUO).

Results. Reverse transcription-polymerase chain reaction (RT-PCR) revealed that, of PADs (type I, II, III, and IV), only PAD type II was expressed in kidneys. Western blot study showed that PAD type II expression and citrullinated protein content increased greatly in kidneys that underwent unilateral ureteral ligation compared to that in contralateral or sham-operated kidneys. Immunohistochemical analyses revealed that PAD type II was preferentially expressed by parietal epithelial cells and that only in Bowman's capsule were proteins citrullinated. Additionally, these PAD type II and citrullinated proteins in obstructed nephropathy were significantly attenuated by the release of the obstruction. Proteome analysis revealed that one of citrullinated proteins in the kidney should be actin.

Conclusion. This result indicates that PAD type II and citrullinated proteins are suitable markers of Bowman's capsule. Not only are these markers preferentially expressed in Bowman's capsules but their expression is also increased in damaged kidneys by UUO, features that promise the further clarification of kidney diseases.

Key words: obstructive nephropathy, Bowman's capsule, parietal epithelial cells, citrullination, peptidylarginine deiminases (PAD), posttranslational modification.

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Bowman's capsule, the outer epithelial wall of the glomerular corpuscle, surrounds glomeruli's loops and lobules. In normal conditions, parietal epithelial cells constituting Bowman's capsule are simple, flat squamous structures. However, the conformation of parietal epithelial cells variously changes in response to such pathologic stimuli as ischemia, hypertension, and inflammation. The result is thickening of the parietal epithelium, and as these parietal epithelial cells proliferate, they become cuboidal and form the glomerular crescents that typify some types of glomerulonephritis. Although Bowman's capsule frequently develops this conformation in patients with kidney diseases, few studies have dealt with it. To date, protein gene product 9.5 is the only known specific marker for Bowman's capsule but this also localizes nerve fibers around arteries [1].

Peptidylarginine deiminase (PADs) are posttranslational modification enzymes that convert protein arginine to citrulline residues [2, 3]. Enzymatic citrullination (deimination) abolishes the positive charge of protein molecules inevitably causing significant alterations in their structure and function [4, 5]. These PADs are distinct from nitric oxide synthetase (NOS) and arginine deiminase, which convert free arginine to citrulline. Mammals have four types of PADs, designated as type I, type II, type III, and type IV [6]. Functionally, PADs have been linked with the pathogenesis of some diseases; for example, PAD type IV is the proposed cause of rheumatoid arthritis and multiple sclerosis [4, 7–9].

Multiple mammalian tissues contain PADs [10] and PAD expression has tissue specificity; for examples, although all PADs are present in epidermis [6], only PAD type II is exclusively expressed in brain [9]. Moreover, citrullination by all PADs shows a definite requirement for Ca^{2+} [11]. Although the mRNA of PAD type II and type IV was previously identified in the rat kidney [6], whether citrullinated proteins also occupy the kidneys

remains uncertain. Therefore, in this study, we used a rat model of kidneys damaged by unilateral ureteral obstruction (UUO) to assess the expression of PADs and the presence of citrullinated proteins. As a result, we present here the first report that specific markers are preferentially expressed in glomerular Bowman's capsules of the kidney and that their expression increases in response to kidney damage.

METHODS

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, USA), weighing 150 to 180 g, were used in this study. Animals were fed standard rodent chow and were given water ad libitum. After intraperitoneal administration of pentobarbital (5.0 mg/kg body weight) (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan), anesthetized animals underwent either left proximal ureteral ligation ($N = 6$) or, simultaneously, a sham operation ($N = 6$). One week later, the obstructed (left UUO) kidneys and the contralateral unobstructed (right) kidneys as well as normal kidneys from sham-operated animals were harvested from these rats. Before removal, the kidneys were well perfused with approximately 100 mL of normal saline to remove circulating blood cell fractions or adherent cells. Blood samples were centrifuged (6000 rpm for 3 minutes) to separate serum for biochemical measurements. Blood urea nitrogen (BUN) and serum creatinine were measured by urease-ultraviolet and enzymatic reaction, respectively (SRL, Inc., Tokyo Japan).

Ureter-bladder anastomosis

In another animal experiment, eight male Sprague-Dawley rats (150 to 180 g) were subjected to left UUO. Four rats of them underwent operation to make ureter-bladder anastomosis 1 week after the ureteral ligation. For building the anastomosis, a sterile and nontoxic polyethylene tube (external diameter 1 mm) (PE-90) (Clay Adams Intradermic Inc., Sparks, MD, USA) was inserted to dilated left ureter and the bladder and fixed securely by silk ligation. These rats were histologically analyzed 5 weeks after the first UUO operation. Simultaneously, the other four rats with UUO for 5 weeks were examined.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The kidney cortex was dissected and homogenized in Isogen (Nippon Gene, Tokyo, Japan) to isolate total RNA, followed by treatment with RNase-free DNase I (Nippon Gene) to remove DNA contamination according to the supplier's protocol. The amount of RNA extracted was estimated by spectrophotometer. RT-PCR was performed with a Takara mRNA Selective PCR Kit (Takara Shuzo Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions. RT was performed using antisense oligonucleotide primers. The primers of rat PADs type I, type II, type II, type IV, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed as described previously [6]. The sense and antisense primers were for rat PAD type I, 5′-AGGTATTAGAA GATGGTGGGGTAGG-3′ and 5′-CCCAACCTTCTC ATCCCCCTTTA-3′ (expected size 631 bp) [12]; for rat PAD type II, 5′-ATTCAAGATAGACCAGGAGGA CCAG-3′ and 5′-CAGAATAGGAAGGCCAGTGTCA GAA-3′ (expected size 428 bp) [10]; for rat PAD type III, 5′- CCTTGGCTTGTGCTTCCTATGGT-3′ and 5′-TCCCTCCCTTCTCCCAGTATGTG-3′ (expected size 648 bp) [6]; for rat PAD type IV, 5′-CGCTC CTGGCAGCCTCCCTCGAGGA-3′ and 5′-CAGCAT CTCTAAGCAGGACTGAGTT-3′ (expected size 205 bp) [12]; and for rat GAPDH, 5′-GTGAAGGTCGGT GTGAACGGAT-3′ and 5′-GCCGCCTGCTTCACCAC CTTCTT-3′ (expected size 788 bp) [6]. We also followed PCR conditions as reported with some modifications [6]; 30 minutes at 50°C, and then 15 minutes at 95°C, followed by 31 or 32 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, and a terminal extension (72°C, 10 minutes). We used the same templates from each experimented kidney for RT-PCR. The condition of templates was checked by amplification for GAPDH. A positive control for RT-PCR of PADs was obtained from rat epidermis where all PADs are expressed [6]. For RT-PCR of PAD type II, mRNA was amplified by mixing primers of PAD type II and GAPDH. PCR products were visualized by agarose gel electrophoresis with ethidium bromide staining. Bands were quantified by densitometry. GAPDH mRNA was analyzed as an internal control, and relative amounts of PAD type II expression were calculated.

Extraction of proteins and Western blotting

Specimens from the renal cortex containing ~500 mg of protein were sonicated in extraction buffer [20 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P40, 50 mmol/L NaF, and 1 mmol/L Na_3VO_4] with protease inhibitor cocktail (Roche, Mannheim, Germany) to extract proteins. Insoluble materials were removed by centrifugation at 15,000 rpm for 10 minutes. The supernatants of samples were incubated with the sample buffer [125 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 0.005% bromophenol blue (BPB), and 0.005% methylene blue] and heated in boiling water for 5 minutes. Protein concentrations were estimated with